Pertanika J. Trop. Agric. Sci. **31(2)**: 247 - 256 (2008) ISSN: 1511-3701

Ultrastructural Studies of Soybean Seed-borne Infection by *Diaporthe phaseolorum* var. *sojae* and Screening of Antagonistic Potentiality by Selected Biocontrol Agents *in vitro*

M.M. Begum[1*], M. Sariah[1], M.A. Zainal Abidin[1], A.B. Puteh[2] and M.A. Rahman[2]
[1]Department of Plant Protection, [2]Department of Crop Science,
Faculty of Agriculture, Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia
**E-mail: miss_mahbuba@yahoo.com*

ABSTRACT

The association of *Diaporthe phaseolorum* var. sojae with soybean seed decay was examined by ultrastructural studies using light microscopy and scanning electron microscopy. The fungus was detected over the seed surface as whitish-grey mycelial growth and scattered black pycnidia. Hyphae and pycnidia were also observed in palisade, hourglass and parenchyma cell layers of the seed coat. Mycelial growth of *D. phaseolorum* var. *sojae* was abundant in the hourglass cell layer compared with other layers of the seed coat. Neither mycelium nor pycnidium was found in any tissues of the cotyledon and embryo of the infected seed. Asymptomatic seeds were free from infections. Artificial seed inoculation with *D. phaseolorum* var. *sojae* significantly reduced seed germination over control by 21.2% and increased seed rot by 120% in sterilized soil under glass house conditions. Six isolates of Trichoderma and three isolates of bacteria were tested in vitro against *D. phaseolorum* var. sojae in dual culture test. Among these organisms, *T. harzianum* isolate UPM40 exhibited the most antagonistic potential based on Percent Inhibition Radial Growth (PIRG) of 92.9% and shortest time needed (7 days) to overgrow the *D. phaseolorum* var. sojae colony as compared to other tested isolates.

Keywords: *Diaporthe phaseolorum*, screening, soybean, biocontrol agents, ultrastructure

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) seed decay caused by *Diaporthe/Phomopsis* complex is one of the major fungal diseases of soybeans in most soybean-producing areas of the world resulting in reduced seed germination and quality (Sinclair, 1988; Sinclair and Backman, 1989; Zorrilla *et al.*, 1994; Fabrega *et al.*, 2000). The most frequently recovered fungus, *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. var. *sojae* (Leh.) Wehm. (anamorph *Phomopsis sojae* Leh.), is the most important causal organism of *Phomopsis* seed decay (Minor *et al.,* 1995). This fungus is seed-borne and responsible for pod and stem blight of soybean. It colonizes both the immature and mature seed tissues of soybean with or without symptoms (Roy and Ratnayake, 1997). Infected seed appears as moldy, smaller in size and fissured which reduces seed quality under warm and humid conditions (Wrather and Sweets 1998; Jackson *et al.*, 2005). *Diaporthe phaseolorum* var. *sojae* is more variable in cultural characteristics and produce typical pycnidia with both α and β conidia (Zhang *et al.*, 1997). A number of practices have been recommended for the control of *Phomopsis* seed decay. Several cultural practices can reduce the level of infection, but not effective enough (Ploper and Backman, 1992). Fungicide has been used extensively but, it is not a widely accepted practice in the tropics, mainly because of its negative effect on the environment and pathogenic resistance (Hartman and Sinclair, 1992). However, control of *Phomopsis* seed decay by genetic resistance or commercial cultivars has not been well explored (Minor *et al.*, 1995; Jackson *et al.*, 2005).

Received: 21 May 2008
Accepted: 2 September 2008
* Corresponding Author

Research on the use of microbial antagonists in biocontrol programs has gained considerable attention for its potential to augment or replace fungicides (Wilson and Wisniewski, 1994). Biological control has been used in many crops to control disease, but the case of soybean is at an embryonic stage (Backman and Jacobsen, 1992). Efforts have been made to use biological control agents to protect seeds from pathogen of soybean. The biocontrol agent *Trichoderma* is well known for having a broad antagonism against different pathogenic fungi in a wide variety of economically important crops (Kloepper *et al.*, 1992; Tronsmo and Hjeljord, 1998; Gardener and Fravel, 2002). Moreover, *Trichoderma* spp. can enhance germination and vigour of poor quality seeds (Harman, 2007). Rhizosphere-colonizing bacteria, such as *Pseudomonas* spp., *Burkholderia* spp., *Bacillus* spp., and *Serratia* spp., are commonly associated with plants in a non-pathogenic manner and are antagonistic against soil-borne plant pathogens (Compant *et al.*, 2005). However, there is no information regarding potential isolates of either *Trichoderma* or rhizobacteria against *D. phaseolorum* var. *sojae*. The aim of this research was to establish the site of infection by *D. phaseolorum* var. *sojae* and its effect on seed germination and seedling survival. Attempts were also made to search for any antagonistic potential of different isolates of *Trichoderma* and rhizobacteria against *D. phaseolorum* var. *sojae in vitro*.

MATERIALS AND METHODS

Microscopic Observations

Ultrastructural studies to determine the site of infection in naturally infected soybean seeds var. Palmetto were conducted in the Plant Pathology Laboratory, Faculty of Agriculture, Universiti Putra Malaysia (UPM) in Serdang, Selangor using light microscopy (LM) and scanning electron microscopy (SEM) through 5 repetitions. Twenty seed samples were randomly selected and plated on moist blotter paper and incubated at alternating cycles of 12 h near-ultra violet (NUV) light and darkness. *Diaporthe phaseolorum* var. *sojae* was identified by cultural and morphological characteristics (Pioli *et al.*, 2003). After four days of incubation, seeds producing fruiting structures of *D. phaseolorum* var. *sojae* as well as asymptomatic seeds were selected. Different seed components viz. seed coat, cotyledon and embryo were separated carefully and cut transversely into 2-3 mm^2 pieces.

Light Microscopy (LM)

Individual seeds and their components from both symptomatic and asymptomatic seeds were fixed in Bouin's solution (picric acid, formaldehyde 40%, glacial acetic acid 15:5:1 v/v/v) and vacuum extracted overnight. Samples were then dehydrated in eight series of concentrations of ethanol (30, 40, 50, 60, 70, 80, 90 and 100%) and washed twice in 98% methyl benzoate plus 2% celloidine for 96 h. Samples were then infiltrated in the oven at 60°C overnight in each of the mixture of xylene and paraplast in proportions of 75:25; 50:50; 25:75; and 100%, respectively. Finally, the samples were embedded in paraffin wax and mounted in small paper boxes. Serial paraffin sections 10-12 µm thick were cut using an ordinary rotary microtome (Model 820 Spencer). Fine sections were placed on glass slides using albumin-glycerine solution. Sections were deparafinized with xylene and stained with 0.05% toluidine blue. All stained sections were mounted in DPX mounting media and viewed under light microscope (Model Nikon FX-35DX) (Johansen, 1940).

Scanning Electron Microscopy (SEM)

Seed samples from symptomatic and asymptomatic seeds were fixed separately in 2.5% buffered glutaraldehyde for 24 h at 4°C. Samples were washed with 0.1 M sodium cacodylate buffer (pH 7.7) and post-fixed in 1 % osmium tetraoxide for 2 h at 4°C and then washed again with 0.1 M sodium cacodylate buffer three times for 10 min each. A series of dehydration was performed in seven different concentrations of ethanol (30, 40, 50, 60, 70, 80 and 90%) three times for 10 min each, and finally for 15 min in 100% ethanol. Samples were dried in Baltec 030 Critical Point Drying (CPD) apparatus for 30 min. Dried samples were stuck on stubs and coated with gold in a Polaron Sputter Coater and viewed under SEM (JOEL JSM 6400) (Benhamou and Chet, 1996).

Fungal Isolation, Seed Inoculation and Disease Assessment

Diaporthe phaseolorum var. *sojae* was isolated from naturally infected soybean seeds by agar plate

method (Begum *et al.*, 2007). The fungus was cultured on PDA for 30 days at room temperature (25±1°C) to induce the formation of pycnidia. The pycnidia were collected and busted with glass rod before washed off with sterilized 1.5% sodium alginate solution. Conidial suspension of α and β conidia obtained was adjusted to a concentration of 1×10^7 conidia ml^{-1} by a Neubeaur haemocytometer. Healthy soybean seeds were surface sterilized with 10% Clorox® for 3 min and rinsed thrice with sterilized distilled water, and dried for 1 h in a laminar flow chamber. Seeds were then soaked in spore suspension (1: 2 w/v) of *D. phaseolorum* var. *sojae* for 1 h and surface dried over night. They were subsequently sowed at the depth of 2 cm in plastic trays (39 x 28 x 11 cm) containing sterilized soil mixture (top soil: peat soil: sand = 3: 2: 1). The experiment was done in four replicates containing 25 seeds each. Trays were arranged in a completely randomized design in the glasshouse with each tray considered as a replicate. The glasshouse temperature fluctuated between 31°C (day) and 25°C (night), and 85 ± 5% R.H. After 14 days, the percentages of seed germination and rotten seed were recorded.

Screening of Biocontrol Agents

Six isolates of *Trichoderma* and three isolates of bacteria obtained from the Plant Pathology Laboratory collection, were used in this study (Table 1). The experiment was conducted using a completely randomized design with five replications and repeated four different times.

These isolates were screened for their antagonistic activity against *D. phaseolorum* var. *sojae in vitro* using dual culture tests. The antagonistic activity was determined based on the Percentage Inhibition of Radial Growth (PIRG). A 5 mm diameter mycelial agar disc was cut from the margin of a 7-day-old culture of *D. phaseolorum* var. *sojae* and placed 3 cm from the edge of a 9 cm Petri dish containing PDA medium. Another 5 mm mycelial agar disc from 7-day-old culture of each *Trichoderma* isolate was placed 3 cm away from the former disc on the same plate. The plates were incubated at ambient temperature (25±1°C) for 15 days. Antagonistic activity of *Trichoderma* isolates were assessed after seven days of incubation by measuring the radius of the *D. phaseolorum* var. *sojae* colony using the following formula (Jinantana and Sariah, 1997):

$$\text{PIRG} = \frac{R1 - R2}{R1} \times 100\,\%$$

where R1 indicates the radial growth of the pathogenic fungal colony in the control plates and R2 indicates the radial growth of pathogenic fungal colony in the dual culture plates. During the incubation period, the time needed for full overgrowth on the colony of *D. phaseolorum* var. *sojae* by each of the *Trichoderma* spp. was recorded up to 14 days. Attempts were also made for the recovery of the fungus from the inhibition and overgrowth zone in the dual culture plates. A 5 mm diameter mycelial agar disc of parasitized fungus by each of the *Trichoderma* spp. was transferred from the inhibition and overgrowth zones on the fresh PDA. The regrowth of the *D. phaseolorum* var. *sojae* was observed after 7 days of incubation.

TABLE 1
Isolates of different biocontrol agents (BCAs)

Isolates	Species
Fungal BCAs	
UPM40	*Trichoderma harzianum*
UPM29	*Trichoderma harzianum*
TL1	*Trichoderma longibrachiatum*
TK1	*Trichoderma koningii*
TV3	*Trichoderma virens*
TV2	*Trichoderma virens*
Bacterial BCAs	
UPM14 B1	*Burkholderia glumae*
UPM13 B8	*Pseudomonas aeruginosa*
UPM39 B3	*Serratia marcescens*

For testing antagonistic bacteria, a 5 mm diameter of fungal agar disc from a 7-day-old culture was placed in the middle of a 9 cm Petri dish containing nutrient agar (NA). The plates were incubated at ambient temperature (25±1°C) for 24 h. A loopful of bacteria from 48 h NA culture was streaked in a circle at 3 cm away from the fungal agar disc. After incubation at room temperature for 7 days, the inhibitory activity of the bacteria was determined by measuring the PIRG and the zone of mycelial growth inhibition around the bacterial streak. Three ratings were used: - = no inhibition zone and growth of fungus over bacterial streak; + = no inhibition zone, but no growth of fungus on the bacterial streak and ++ = 1-5 mm inhibition zone (Bardin *et al.*, 2004).

Statistical Analysis

Data were analyzed statistically by ANOVA using SAS software (SAS, 1999). Mean separation was carried out using Tukey's Studentized Range (HSD) at $P = 0.05$.

RESULTS

Ultrastructural studies of asymptomatic and symptomatic soybean seeds infected by *D. phaseolorum* var. *sojae* conducted under LM and SEM revealed no fungal propagule externally and internally for asymptomatic seeds (*Figs. 1A* and *B*). Seeds showing symptoms of *D. phaseolorum* var. *sojae* infection indicated a profuse mycelial growth with black pycnidia over the seed surface. The seeds appeared externally white, chalky and shriveled (*Figs. 2A-B, 3A* and *4A*). Pycnidial beak was found in severely infected seeds (*Fig. 2B*). Mycelia and pycnidia of *D. phaseolorum* var. *sojae* were observed in all layers of the seed coat. Fungal hyphae could be distinguished in the seed tissue based on hyphal morphology. Hyphae of this fungus were hyaline, branched and stained light green with toluidine blue (0.1%). The hyphal breadth ranged from 3.5- 7.5 µm (*Figs. 3B* and *4B*). Pycnidia were found in the palisade cell layer, hourglass cell layer and parenchyma cell (*Figs. 3B-D* and *4C*). Mycelial growth was more abundant in the hourglass layer but less in parenchyma and palisade cell layer of the seed coat (*Figs. 3B* and *4B*). Hyphae and pycnidia were not detected in any tissue of the cotyledons or embryo of infected seeds.

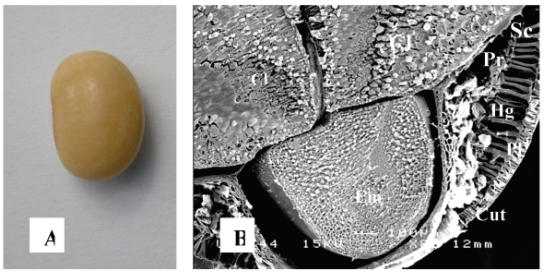

Fig. 1: Photomicrographs showing asymptomatic soybean seeds (A) External view of seed under LM (B); Transverse section of internal seed tissues under SEM (Abbreviations: Sc, Seed coat; Cl, Cotyledon; Em, Embryo; Cut, Cuticle; Pl, Palisade cell; Hg, Hourglass cell; Pr, Parenchyma cell)

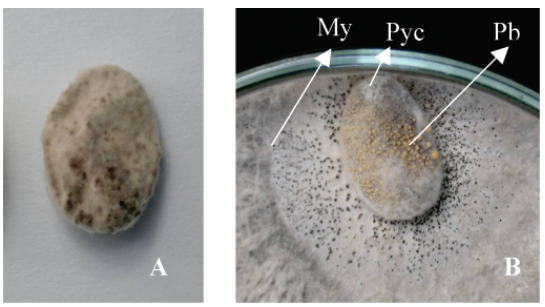

Fig. 2: Light microscopic photomicrographs showing naturally infected soybean seeds by D. phaseolorum *var.* sojae *with grayish-white moldy and scattered black pycnidia over seed surface (A) on moist blotter paper; (B) on PDA (My, Mycelia; Pyc, Pycnidia and Pb, Pycnidial beak)*

The effect of seed inoculation with *D. phaseolorum* var. *sojae* was evaluated on seed germination, and rot of soybeans under glass house conditions (Table 2). Inoculated seeds resulted in lower seed germination at 67.0%, whereas 85.0% was recorded in uninoculated seeds. The results obtained clearly indicated that the fungus reduced seed germination significantly (P=0.05) by 21.2% in comparison with uninoculated seeds (control). A higher percentage (33.0%) of seed rot was also recorded in inoculated seeds than that of uninoculated seeds (15.0%), which corresponded to an increasing seed rot by as much as 120%.

All *Trichoderma* isolates were found to inhibit the radial growth of *D. phaseolorum* var. *sojae* at different degrees of inhibition (Table 3 and

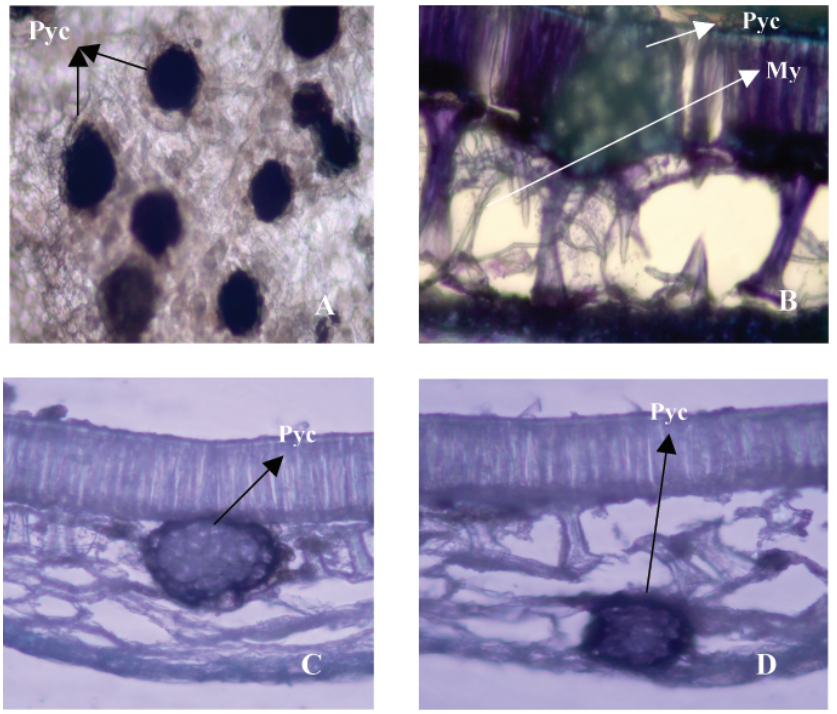

Fig. 3: Light microscopic photomicrographs showing mycelia and pycnidia of D. phaseolorum *var.* sojae *on and in the seed coat of soybean seeds (A); Black pycnidia and mycelia on the seed coat (B); Pycnidia in palisade layer and mycelial growth in the internal layer of the seed coat; (C) Pycnidia in hourglass cell of the seed coat (D) Pycnidia in parenchyma cell of the seed coat (Abbreviations: Pyc, Pycnidia; My, Mycelia)*

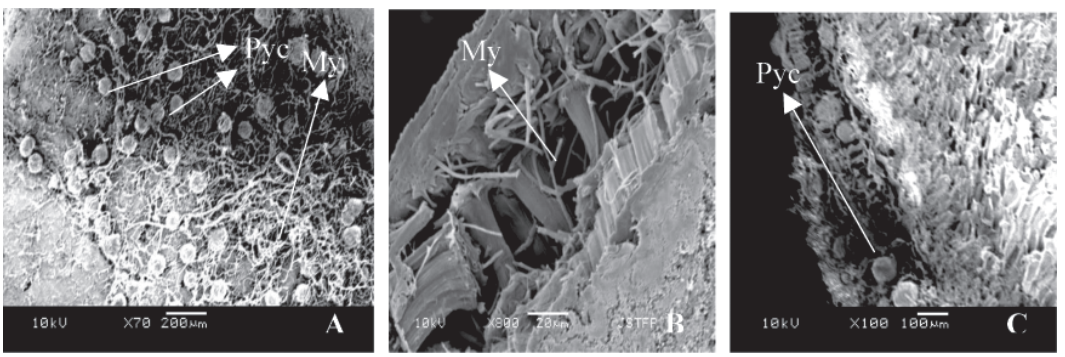

Fig. 4: Scanning electron micrographs (SEM) showing mycelia and pycnidia of D. phaseolorum *var.* sojae *on and in the seed coat in naturally infected soybean seeds (A) Black pycnidia and mycelia on the seed coat (B) Profuse mycelial growth in the internal layer of the seed coat (C) Pycnidia in all layer of the seed coat (Abbreviations: Pyc, Pycnidia; My, Mycelia)*

Fig. 5). The highest PIRG value recorded was UPM40 (92.9%) followed by TV2 (74.5%), TL1 (73.9%), TV3 (71.3%), TK1 (59.9%) and UPM29 (51.9%). The time needed for overgrowth on the pathogenic fungal colony by UPM40 was shortest as compared to other *Trichoderma* isolates. UPM40 completely grew over the fungal pathogen colony 7 days after incubation. The fungal pathogen failed to re-grow when colonized by all *Trichoderma* spp. on fresh PDA (*Fig. 6*). No

TABLE 2
Effect of seed inoculation by *D. phaseolorum* var. *sojae* on seed germination and seed rot of soybean

Treatment	Seed germination (%)	Reduction of germination over control (%)	Seed rot (%)	Increased seed rot over control (%)
Inoculated seed	67.00 b	21.2	33.00 c	120
Uninoculated seed (Control)	85.00 a	0.0	15.00 d	0.0

Means within the same column followed by the same letter are not significantly different at P=0.05 according to Tukey's Studentized Range (HSD) performed on arcsine transformed data.

TABLE 3
Antagonistic effect of *Trichoderma* isolates against *D. phaseolorum* var. *sojae* in the dual culture test

Isolates code no.	Genus/Species name	Antagonism (PIRG)*	Time of overgrowth
UPM40	*Trichoderma harzianum*	92.9 a	7-days
UPM29	*T. harzianum*	51.9 c	-
TL1	*T. longibrachiatum*	73.9 b	9-days
TK1	*T. koningii*	59.9 bc	-
TV3	*T. virens*	71.3 b	11-days
TV2	*T. virens*	74.5 b	9- days

- indicates no overgrowth up to 14 days
* indicates percent inhibition of radial growth (PIRG) at 7 days after incubation
Means within the same column followed by the same letter are not significantly different at P=0.05 according to Tukey's Studentized Range (HSD) performed on arcsine transformed data.

bacterial isolates showed potential to control *D. phaseolorum* var. *sojae* in the dual culture test (Table 4 and *Fig.* 7). They exhibited slight inhibition (+), which was less then 50% after 7 days of incubation. There was no inhibition zone formed between bacterial isolates and *D. Phaseolorum* var. *sojae*.

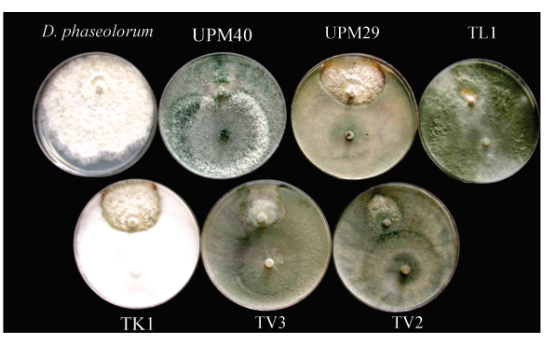

Fig. 5: Radial growth of D. phaseolorum *var.* sojae *in the dual culture test as affected by* Trichoderma *spp, seven days after incubation on PDA*

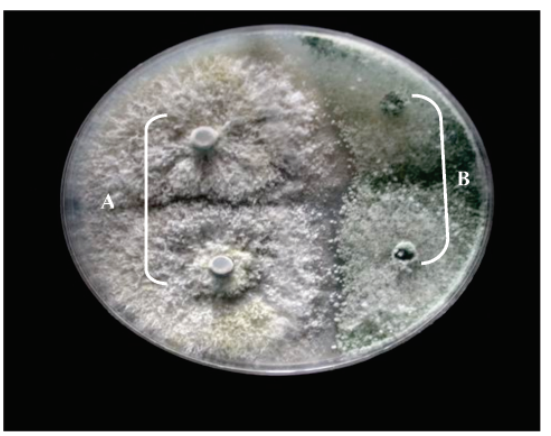

Fig. 6: Regrowth of D. phaseolorum *var.* sojae *in the dual culture test as affected by* Trichoderma *spp, seven 7 days after incubation on PDA. (A)* D. phaseolorum *var. sojae from the control plate; (B) there was no recovery of* D. phaseolorum *var. sojae from the overgrowth zone and interaction zone in the treatment plate*

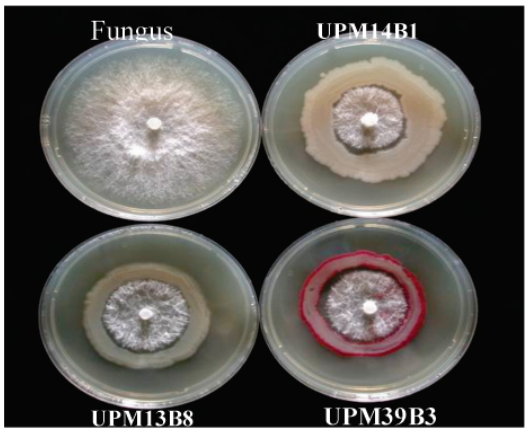

Fig. 7: Radial growth of D. phaseolorum *var.* sojae *in the dual culture test as affected by bacterial isolates, seven days after incubation on NA*

DISCUSSION

Ultrastructure views of symptomatic soybean seeds by *D. phaseolorum* var. *sojae* revealed the colonization of mycelia and pycnidia on and in all layers of the seed coat. The result confirmed that the fungus was an externally and internally seed-borne pathogen and could remain dormant and as latent infection (Sinclair, 1991). Mycelial growth was much more prevalent in the hourglass cell layer than other closely compacted layers of the seed coat. This may be attributed to the large intercellular spaces available that allow for more growth of fungal pathogen in the hourglass cell (Kunwar *et al.*, 1985). Similar trends in the spread of mycelium of *D. phaseolorum* var. *sojae* was observed in the seed coat of infected soybean seeds, but occasionally found in embryonic and cotyledonary tissues (Ilyas *et al.*, 1975). Singh and Sinclair (1986) observed the mycelium of *Phomopsis* sp. on the surface, in all layers of seed coat and the embryo (cotyledons). Kunwar *et al.* (1985) observed that *Phomopsis* spp. colonized seed coat and embryo tissues in mixed infected soybean seeds with *Colletotrichum truncatum*. *Phomopsis* was located in the seed-coat with a relatively low incidence in the embryo tissue (Zorrilla *et al.*, 1994). Spread of the pathogen by fungal propagule in infected seed tissues depended on the severity of infections, seed stage and genotypes (Singh and Mathur, 2004).

Results from the glass house study demonstrated that *D. phaseolorum* var. *sojae* caused seed rot of soybean. The germination of infected seeds was found to be significantly (P=0.05) lower (21.2%) when compared with the uninoculated seeds. *Diaporthe phaseolorum* var. *sojae* was found to be associated with soybean seeds infection both externally and internally of the seed coat which lead to local and systemic infections. Thus, infected seeds reduced germination by progressive rotting of the hypocotyl-radicle axis of soybeans (Vrandecic *et al.*, 2006). Kmetz *et al.* (1978) reported that *D. phaseolorum* var. *sojae* negatively affected seed germination and caused seed rot. Vrandecic *et al.* (2006) found *Phomopsis sojae* to be the most pathogenic to soybean seeds among twelve isolates of *Diaporthe/Phomopsis* species. This fungus caused seed rots after artificial seed inoculation.

In vitro screening is considered the most desirable first step to screen for a large number of potential antagonists in a biocontrol process for possible application in the field (Merriman and Russell, 1990). The preliminary screening of different isolates of *Trichoderma* and bacteria was conducted using dual culture test *in vitro*. *Trichoderma harzianum* (UPM40) showed the highest antagonistic potential to suppress the growth of *D. phaseolorum* var. *sojae* based on higher PIRG value and time needed for complete

TABLE 4
Antagonistic effect of bacterial isolates against *D. phaseolorum* var. *sojae* in the dual culture test

Isolates code no.	Genus/Species name	Antagonism (PIRG)*	Inhibition category*
UPM14 B1	*Burkholderia glumae*	33.9 a	+
UPM13 B8	*Pseudomonas aeruginosa*	33.1 a	+
UPM39 B3	*Serratia marcescens*	34.1 a	+

* indicates percent inhibition of radial growth (PIRG) at 7 days of incubation

Means within the same column followed by the same letter are not significantly different at *P*=0.05 according to Tukey's Studentized Range (HSD) performed on arcsine transformed data.

overgrowth compared to other *Trichoderma* isolates. However, the tested bacterial isolates did not show any prospective inhibitory effect to *D. phaseolorum* var. *sojae*. Although, PIRG values of all bacterial isolates indicated positive results, the values were less than 75%. A PIRG value of more than 75% is required to be considered as a potential antagonist (Narayanasamy, 2006). These bacterial isolates were isolated from oil palm roots and tested for their antagonistic activity against *Ganoderma boninense*, a soilborne pathogen (Zaiton, 2006). Specificity of the bacterial isolates could lead to the low *in-vitro* effectiveness against *D. phaseolorum*. The time needed for overgrowth on the pathogen colony is an important parameter in the assessment of the antagonistic ability to compete against the pathogen for limited nutrient resources and space (Ibrahim, 2005). *Trichoderma harzianum* was able to completely overgrow the fungal pathogen colony within 7 days. There was no recovery of *D. phaseolorum* var. *sojae* from the parasitized mycelia by *Trichoderma* spp. Fernandez (1992) reported that application of *T. harzianum* to soybean residues resulted in a significant decrease in the incidence of soybean pathogens. In field plots, *T. harzianum* increased soybean plant survival by 40% (Menendez and Godeas, 1998). The inhibition of radial growth of fungal mycelia in this study was considered to be either competition or mycoparasitism or antibiosis which inhibited fungal pathogen activity and caused their lyses (Wilson and Wisniewski, 1994; Harman, 2005). The activity of *T. harzianum* in this study suggested that it can be used as a potential antagonist to suppress *D. phaseolorum* var. *sojae* of soybean in the field. Further research is needed to develop formulation and the most suitable technique for field application of biocontrol agents in comparison to recommended fungicides.

ACKNOWLEDGEMENT

The authors are sincerely grateful to the Third World Organization for Women in Science (TWOWS) for the financial support to conduct this research at Universiti Putra Malaysia.

REFERENCES

Backman, P.A. and Jacobsen, B.J. (1992). Soybean disease management: chemical and biological control in temperate regions. In L.G. Copping, M.B. Green and R.T. Rees (Eds.), *Pest managemant in soybean* (p. 155-163). London and New York: SCI: Elsevier Applied Science.

Bardin, S.D., Huang, H., Pinto, J. Amundsen, E.J. and Erickson, R.S. (2004). Biological control of *Pythium* damping-off of pea and sugar beet by *Rhizobium leguminosarum* bv. *Viceae*. *Can. J. Bot.*, *82,* 291-296.

Begum, M. M., Sariah, M., Puteh, A.B. and Zainal Abidin, A. A. (2007). Detection of seed-borne fungi and site of infection by *Colletotrichum truncatum* in naturally-infected soybean seeds. *Inter. J of Agric. Res.*, *2,* 812-819.

Benhamou, N. and Chet, I. (1996). Parasitism of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum* and *Rhizoctonia solani*: Ultrastructural and cytochemical aspects of the interaction. *Phytopathol.*, *86,* 405-416.

Compant, S., Duffy, B., Nowak, J., Clement, C. and Barka, E.A. 2005. Use of plant growth promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *App. and Environ. Microb.*, *71,* 4951 - 4959.

Fabrega, A.R., Lamarque, A.L., Giorda, L.M., Guzman, C.A. and Maestri, D.M. (2000). Effect of *Diaporthe phaseolorum* var. *sojae* infection on seed quality of soybean genotypes. *Fitopatol., Bras.*, *25,* 624-627.

Fernandez, M.R. (1992). The effect of *Trichoderma harzianum* on fungal pathogens infesting soybean residues. *Soil Biol. Biochem.*, *24,* 1027-1029.

Gardener, M.B.B. and Fravel, D.R. (2002). Biological control of plant pathogens: Research, Commercialization and application in the USA. *Plant Health Progress* (Online), DOI:10.1094/PHP-2002-0510-01-RV.

Harman, G.E. (2005). Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathol.*, *96,* 190-194.

Harman, G.E. (2007). Plant productivity enhancement by biological seed treatments. Seed testing Int., *ISTA News Bull.*, *133,* 15.

Hartman, G.L. and Sinclair, J.B. (1992). Soybean disease management: chemical and biological control in tropical regions. In

L.G. Copping, M.B. Green and R.T. Rees (Eds.), *Pest managemant in soybean* (p. 164-173). London and New York: SCI: Elsevier Applied Science.

Ibrahim, M.D. (2005). Delivery system of Trichoderma inoculant for the control of Rhizoctonia diseases in *Brassica rapa* (PhD Thesis, Universiti Putra Malaysia, Serdang, Selangor).

Ilyas, M.B., Dhingra, O.D., Ellis, M.A. and Sinclair, J.B. (1975). Location of mycelium of *Diaporthe phaseolorum* var. *sojae* and *Cercospora kikuchii* in infected soybean seeds. *Plant Dis. Rep.*, *59,* 17-79.

Jackson, E.W., Fenn, P. and Chen, P. (2005). Inheritance of resistance to *Phomopsis* seed decay in soybean PI 80837 and MO/PSD-0259 (PI 562694). *Crop Sci.*, *45,* 2400-2404.

Jinantana, J. and Sariah, M. (1997). Antagonistic effect of Malaysian isolates of *Trichoderma harzianum* and *Gliocladium virens* on *Sclerotium rolfsii. Pertanika J.of Trop. Agric. Sci., 20,* 38-41.

Johansen, D.A. (1940). *Plant Microtechnique.* New York: McGraw-Hill.

Kloepper, J.W., Schippers, B. and Bakker, P.A.H.M. (1992). Proposed elimination of the term endorhizosphere. *Phytopathol.*, *82,* 726-727.

Kmetz, K.T., Schmitthenner, A.F. and Ellett, C.W. (1978). Soybean seed decay: prevalence of infection and symptom expression caused by *Phomopsis* spp. *Phytopathol.*, *68,* 836-840.

Kunwar, I.K., Singh, T. and Sinclair, J.B. (1985). Histopathology of mixed infections by *Colletotrichum truncatum* and *Phomopsis* spp.or *Cercospora sojina* in soybean seeds. *Phytopathol.*, *75,* 489-492.

Menendez, A.B. and Godeas, A. (1998). Biological control of *Sclerotium sclerotiorum* attacking soybean plants: Degradation of the cell walls oh this pathogen by *Trichoderma harzianum* (BAFC 742). *Mycopathol.*, *142,* 153-160.

Merriman, P. and Russell, K. (1990). Screening strategies for biological control. In D. Hornby (Ed.), *Biological control of soil-borne plant pathogens* (p. 427–435). Wallingford, UK: CAB Int.

Minor, H.C., Brown, E.A. and Zimmerman, M.S. (1995). Developing soybean varieties with genetic resistance to *Phomopsis* spp. *J. Amer. Oil Chemist Soc.*, *72,* 1431-1434.

Narayanasamy, P. 2006. *Postharvest Pathogens and Disease Management.* John Wiley & Sons, Inc.

Pioli, R.N., Morandi, E.N. Martinez, M.C. and Lucca, F. (2003). Morphologic, molecular, and pathogenic characterization of *Diaporthe phaseolorum* variability in the core soybean-producing area of Argentina. *Phytopathol.*, *93,* 136-146.

Ploper, L.D. and Backman, P.A. 1992. Nature and management of fungal diseases affecting soybean stems, pods and seeds. In L.G. Copping, M.B. Green and R. T. Rees (Eds.), *Pest managemant in soybean* (pp. 174-184). London and New York, SCI: Elsevier Applied Science.

Roy, K.W. and Ratnayake, S. (1997). Frequency of occurrence of *Fusarium pallidoroseum*, effect on seeds and seedlings and association with other fungi in soybean seeds and pods. *Can. J. Plant Pathol.*, *19,* 188–192.

SAS (1999). *SAS/STAT Guide to Personal Computer*, Version 8.1, SAS Institute Inc., Cary, North Carolina, USA.

Sinclair, J.B. and Backman, P.A. (1989). *Compendium of Soybean Diseases* (3rd edn.) St. Paul, Minnesota: American Phytopathol. Soc.

Sinclair, J.B. (1988). *Diaporthe/Phomopsis* complex of soybeans. In T.D. Wyllie and D.H. Scott (Eds.), *Soybean diseases of the north central region* (p. 96-101). St Paul, Minnesota: American Phytopathol Soc.

Sinclair, J.B. (1991). Latent infection of soybean plants and seeds by fungi. *Plant Dis.*, *75,* 220-224.

Singh, D. and Mathur, S.B. (2004). *Histopathology of Seed-borne Infections.* Washington DC: CRC press.

Singh, T. and Sinclair, J.B. (1986). Further studies on the colonization of soybean seeds by *Cercospora kikuchii* and *Phomopsis* spp. *Seed Sci. Technol.*, *14,* 71-77.

TRONSMO, A. and HJELJORD, L.G. (1998). Biological control with *Trichoderma* species. In G.S. Boland and L.D. Kuykendall (Eds.), *Plant-microbe interactions and biological control* (p.111-124). New York: Marcel Dekker.

VRANDECIC, K.D., JURKOVIC, D. and COSIC, J. (2006). Effect of *Diaporthe/Phomopsis* species isolated from soybean and *Abutilon theophrasti* on soybean seed germination. *J. Phytopathol.*, *154,* 725-728.

WILSON,C.L. and WISNIEWSKI, M.E. (1994). *Biological Control of Postharvest Diseases of Fruits and Vegetables- Theory and Practices.* Boca Raton, FL: CRC Press.

WRATHER, J.A. and SWEETS, L.E. (1998). Soybean disease management. http://muextension.missouri.edu/xplor/agguides/crops/g04452.htm.Cited on 27 Jan 2006.

ZAITON, S., M. SARIAH and M. A. ZAINAL ABIDIN. (2008). Effect of endophytic bacteria on growth and suppression of *Ganoderma* infection in oil palm. *Inter. J. Agric.e and Biol., 10,* 127-132.

ZHANG, A.W., RICCIONI, L., PEDERSON, W.L., KOLLIPARA, K.P. and HARTMAN, G.L. (1997). Molecular identification and phylogenetic grouping of *Diaporthe phaseolorum* and *Phomopsis longicola* isolates from soybean. *Phytopathol.*, *88,* 1306-1314.

ZORRILLA, G., KNAPP, A.D. and MCGEE, D.C. (1994). Severity of *Phomopsis* seed decay, seed quality evaluation and field performance of soybean. *Crop Sci.*, *34,* 172-177.